

A CELL-MEMBRANE BOUND FRACTION OF BACTERIAL DNA

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In contrast to the abundance of information on the enzymatic replication of DNA in vitro (Kornberg, 1962), the organization and replication of the bacterial chromosome is not well understood. Genetic and autoradiographic studies suggest a model of the bacterial genome as a closed ring, only a small critical region of which is being replicated at any given time (Sueoka, 1963; Cairns, 1963; Nagata, 1963; Lark and Lark, 1964; Hanawalt and Ray, 1964). Jacob, et. al. (1963), proposed a model in which replication might occur in direct association with the cell membrane. There is no direct evidence for the site of DNA replication in the cells, although Lark and Lark (1964) suggested the possible involvement of some structural protein cell wall material in the regulation of chromosome replication. However, many relevant facts have been reported, mostly concerning the localization of nascent DNA, i.e., DNA which is enriched in counts from a recent pulse of labeled precursors.

Goldstein and Brown (1961) using E. coli protoplasts, showed the occurrence of DNA synthesis in a sedimentable heavy particle fraction which was resistant to sonic oscillation and rich in nascent DNA. Ben-Porat, et. al. (1962), found that nascent DNA from mammalian cells was preferentially agglomerated at an interface during deproteinization. DNA-polymerase complexes in E. coli lysates were reported by Billen (1962) and Kadoya, et. al. (1964). In B. subtilis, Ganesan (1963) found the total DNA of cells could be pelleted after controlled lysis; the complex had a molecular weight in the range of 50-70 million.

Smith (1964), found that in *E. coli* lysed by detergent, 50% of nascent DNA sedimented at low centrifugal speeds (2000 rpm) and was bound to the particulate fraction. Electron micrographs of *B. subtilis* show cellular DNA associated with cell membrane (Ryter and Jacob, 1963; Ryter and Landman, 1964). Hanawalt and Ray (1964), found that nascent DNA in *E. coli* was difficult to extract without treatment with proteolytic enzymes. Detergent and phenol treatments discriminated against newly replicated DNA. The nascent DNA containing fractions were pycnographically less dense, suggesting a protein complex. A preliminary account of the following results have been reported earlier (Ganesan and Lederberg, 1965).

Materials and Methods

Stocks of labeled precursors as purchased were:

Deoxythymidine-2-C¹⁴, New England Nuclear,
30 mc/mM (0.807 mg/ml in H₂O).

Deoxythymidine methyl-H³, New England Nuclear,
6.7 C/mM (0.18 mg/5ml in H₂O).

Deoxyadenosine triphosphate, H³ (NH₄)₄ Schwartz BioResearch
1.2 C/mM.

A thymine requiring strain of *B. subtilis* (Thy⁻ Try⁻) (kindly given by Dr. F. Rothman), which is kept in our stocks as SB 566, was used in these experiments. A culture was grown for 3 generations (150 minutes doubling time) in minimal medium¹ containing 10 µg/ml of DL-tryptophan and 21 µg/ml of C¹⁴-deoxythymidine having a specific activity of 8×10^3 CPM/µg in our counter. The DNA from such a culture at the end of 3 generations had a specific activity of 600 CPM/µM. The labeled culture was washed two times and resuspended in thymine-free minimal medium at a concentration of 4×10^9 cells/ml and incubated at 37°C. for 10 minutes

¹ Spizizen's minimal medium with 0.02% caesin hydrolysate (ref. 6).

to deplete the internal thymine pool (no inviability was noted before 15 minutes). Deoxythymidine methyl- H^3 (.01 mc) was added to 2×10^{10} cells in 5 ml for different lengths of time. The growth was stopped by addition of azide buffer¹. 3×10^8 cells in 30 λ , having 2.14 μ M of DNA, were lysed with 50 μ g of lysozyme followed by addition of 0.27 ml of the same buffer without EDTA. At this point, sodium dodecyl sulfate was added to 0.1% concentration. The cleared lysate was layered on a sucrose gradient (Burgi and Hershey, 1963), and spun at 36,000 rpm for 3 hours at 10°C. At the end of this treatment less than 10^{-5} of the cells were viable. For polymerase assay, the same procedure was used without detergent but with control of lysis under the microscope. The lysis without detergent takes longer at 37°C. with the same residual viability. Mg^{++} (10^{-2} M) was essential for the stability of sedimentable polymerase membrane complex, as well as for its activity in the assay (Fig. 4). DNA polymerase was assayed (Richardson, *et. al.*, 1964), using dAT*polymer as a primer with two added triphosphates of deoxyadenosine and deoxythymidine. The dATP H^3 was diluted to give 1.5×10^5 cpm/10 μ M of dATP.

The backgrounds for the gradients were determined by running an acid soluble supernatant in a sucrose gradient, followed by acid precipitation with added carrier. In general, a small proportion of acid precipitable C^{14} and H^3 counts were found on the top of the gradient. They were biologically inactive, presumably oligonucleotides. The nascent DNA of cultures approaching the stationary phase is unstable in lysates giving rise to acid soluble counts on standing. In the reported pulse experiments, we have lost from 8 to 20% of the nascent DNA, while the non-replicating DNA was completely recovered. (These

¹ 10^{-2} M sodium azide, 10^{-2} M Tris-HCl, pH 7.8, 10^{-2} M $MgCl_2$, 10^{-2} M EDTA and 10^{-1} M NaCl. (0°C)

* copolymer of deoxyadenylate and deoxythymidylate.

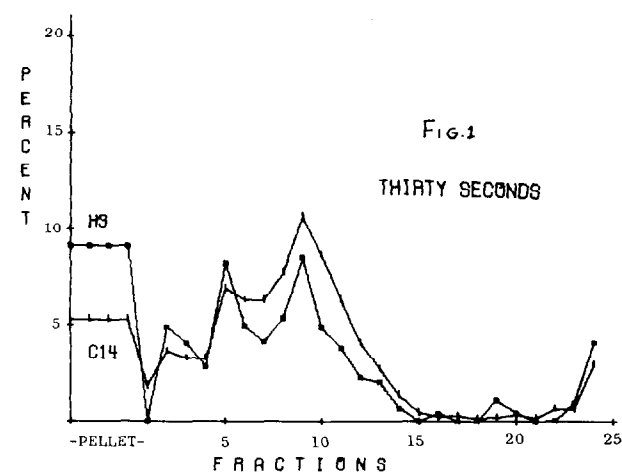
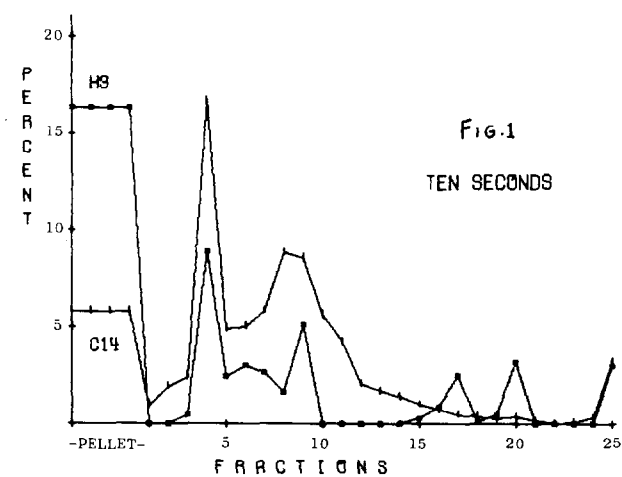
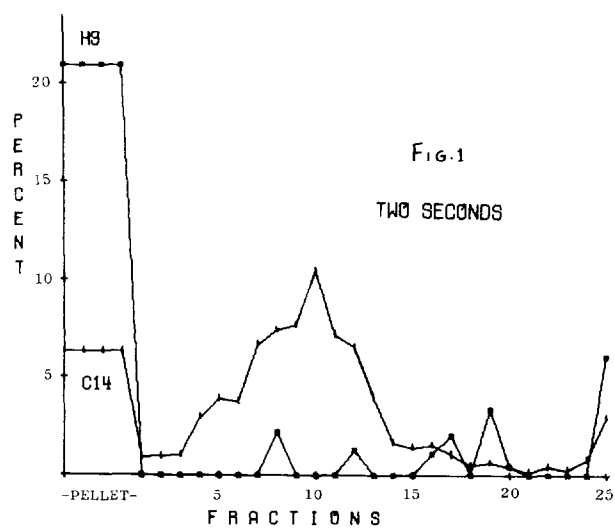


Figure 1.

Sucrose gradient sedimentations of lysates of pulse-labeled cells; thymidine- H^3 added for 2, 10 and 30 seconds, respectively. The pellet is represented as if it were evenly distributed among 4 fractions. Each fraction consists of 3 drops (.25 ml). The distributions are normalized as percentages of the total counts, as given below.

Time of Pulse	Total H^3 CPM	Total C^{14} CPM	μ M of DNA	μ M of New DNA
2"	223	1.2×10^3	2.14	1.3×10^{-4}
10"	426	1.2×10^3	2.14	2.6×10^{-4}
30"	876	1.2×10^3	2.14	5.1×10^{-4}

Time of Pulse	$H^3\%$ in Pellet	$C^{14}\%$ in Pellet	$H^3\%$ in the Sup.	$C^{14}\%$ in the Sup.
2"	83.68	25.38	16.32	74.62
10"	65.30	23.07	34.7	76.93
30"	36.37	21.06	63.63	78.94

The same results were obtained using up to 5x the amount of DNA and H^3 pulse counts. In 8 experiments, the shortest pulse always yielded from 68-96% of the nascent DNA in the pellet. The above set is given because it was done at the same time with the same cells and in the same gradient run, so all the conditions are comparable with little variation. In these 8 experiments, the bulk C^{14} labeled non-replicating DNA varied from 20-60% in the pellet. It is difficult to predict the amount of non-replicating DNA in the pellet while the behavior of nascent DNA is easily predicted. (Sup. refers to supernatant.)

The plot is computer-generated (on a Calcomp Digital Plotter reading IBM 7090 output).

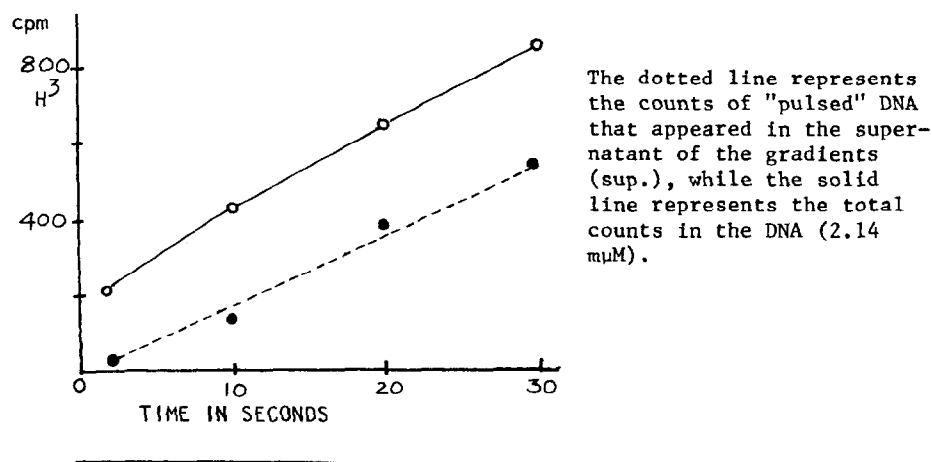
observations suggest either that the nascent DNA might be more susceptible to nucleases or perhaps more likely, that the nucleases are also localized in the sedimentable fractions.)

Differential counting was done using a Packard TriCarb Liquid Scintillation Counter. In general, low amounts of lysate gave very reproducible results with no aggregation effects. Transformation assays were performed as described by Ganesan and Lederberg (1964).

Results

Figure 1 shows that the nascent DNA (H^3 labeled), is predominantly associated with the pellet fraction. Electron microscope studies indicated predominantly membranes and broken cell wall structures in the pellet. Figure 2 shows the H^3 counts incorporated during different lengths of "pulses", and suggests a constant rate of DNA synthesis after the first datum (2 seconds). The initial rate may show an error in the time base of 9 seconds, perhaps due to delay in stopping the growth. The absolute amount of non-nascent DNA found in the pellet varied considerably from experiment to experiment. If replication is localized, then the separation of nascent DNA from the chromosome depends on the distribution of breaks which depends on capricious fluid shear. Very careful lysis with no agitation and no pipetting gives lysates from which all the DNA sediments out as pellet.

Figure 2. Incorporation of H^3 thymidine by 3×10^8 cells.



Microscopic examination during the process of lysis reveals that lysozyme attacks the cell wall at irregular intervals. The cells become fragile and in the absence of stabilizing agents the bulk of the cytoplasm and some RNA leaches out of the ruptured membrane. We

interpret this to mean that the DNA still attached to the membrane is protected by it and sediments easily. This might be the mechanism of "nuclear body" formation which was observed by Spiegelman, *et. al.* (1958). The chemical composition of this pellet (Ganesan, 1963), is similar to that found in *B. megatherium* by Spiegelman, *et. al.* (1958) and Butler and Godson (1963).

Chase Experiments

If the replicating point(s) were attached to a specific site on the membrane cell wall, it should be possible to chase the "pulse" label

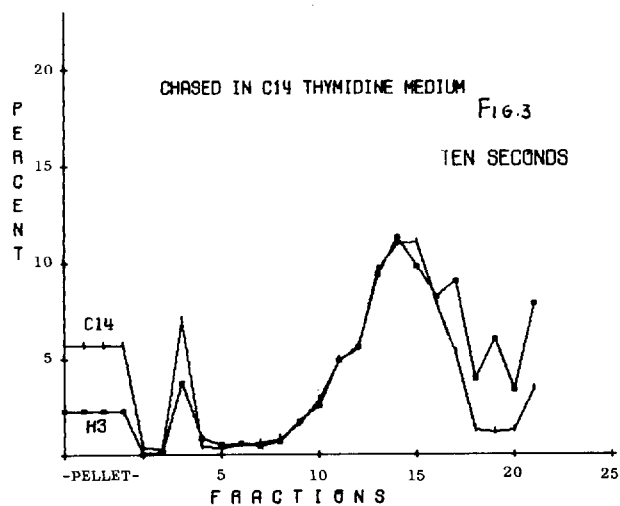
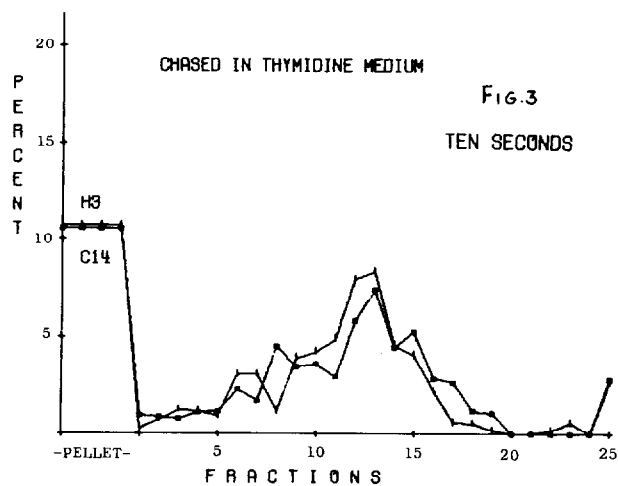


Figure 3.

H³ pulse chased in C¹⁴ thymidine medium.

	<u>Pellet</u>	<u>Sup.</u>
C ¹⁴	24%	76%
H ³ Pulse	9.06%	90.94%
(H ³ 100% = 4.6×10^3 CPM while C ¹⁴ = 1×10^4 CPM.)		

H³ pulse chased out in cold thymidine medium.

	<u>Pellet</u>	<u>Sup.</u>
C ¹⁴	43%	57%
H ³	42.4%	57.6%
(H ³ 100% = 8.7×10^3 CPM while C ¹⁴ = 1.7×10^3 CPM.)		

These experiments were also tried with different amounts of DNA per gradient. More than 10 μ M of DNA in a 5 ml gradient (approx. 2×10^9 cell equivalents) leads to aggregation so that bulk of DNA sediments out as a gel in the pellet.

from the pellet by subsequent growth in cold thymidine, as indicated by a uniform ratio of H³/C¹⁴ constant throughout the gradient. If the H³ pulse were chased by subsequent growth in C¹⁴ thymidine medium, the ratio of H³/C¹⁴ should change drastically, especially in the pellet. These results are shown in Figure 3 for 10" pulse.

DNA Polymerase Activity

Cells labeled uniformly with C¹⁴ thymidine were used in this experiment (Figure 4). The DNA polymerase complex is Mg⁺⁺ dependent. The pellet contains 1/3 of the DNA. The fractions most active in polymerase are in the pellet and top of the gradient. Treatment of the pellet with pronase or omitting one of triphosphates or Mg⁺⁺ resulted in loss of activity. Under these conditions the cellular DNA did not have any inhibitory effect on the synthetic ability with dAT³²P as primer. The

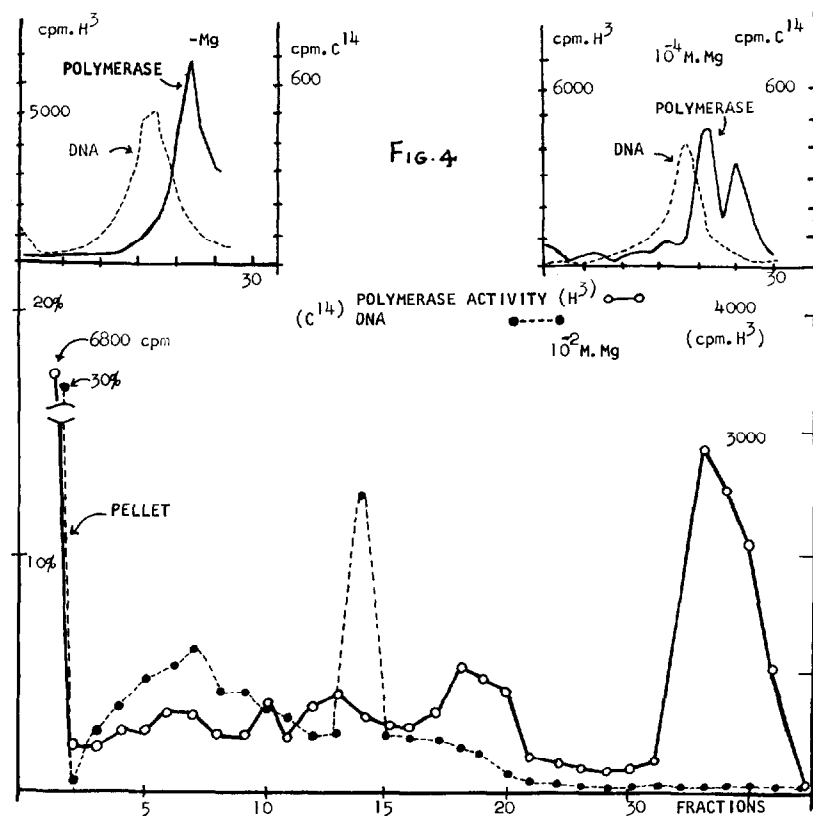


Figure 4.

DNA polymerase complexes. The gradients were run with 10^{-4} M β -mercaptoethanol. Mg^{++} was added as indicated. The inserts show the effect of Mg^{++} at 0 and 10^{-4} M while the main figure shows the effect at 10^{-2} M concentrations. Note the pellet has no enzymatic activity or appreciable amount of DNA at 0 and 10^{-4} M Mg^{++} concentrations. In all these cases residual unlysed cells were removed by centrifugation prior to zone sedimentation. 100% corresponds to 3×10^4 CPM per 5 μ moles of DNA and 12 μ g of protein was added per gradient. In the insert figures, the specific activity of DNA was less while the concentrations are approximately the same. 50 λ of each fraction was assayed for polymerase.

results of detailed kinetic experiments are under publication (Ganesan, *et. al.*, 1965). From these experiments we conclude that the membrane fraction possesses up to 25% of the total assayable DNA polymerase activity.

Discussion

B. subtilis has a genome of 1.8×10^6 nucleotide pairs. Under these conditions with a generation time of 150 minutes, 1.2×10^4 nucleotide pairs replicates every minute. When the cells are washed and starved as in these "pulse" experiments, the replication is very slow. The slope of figure 2 indicates a rate of the order of 5.7 times slower than the normal replication rate. This may be due to the washing procedures introduced before exposure to H^3 thymidine. The nascent DNA, after 2 seconds exposure to H^3 thymidine, bands in CsCl gradient at a stratum of 1.703 gms/cc.

As far as can be judged from the limited data of figure 2, i.e., the displacement of the 2 curves, the transit time of nascent DNA is about 10.5 seconds, corresponding to 168-200 nucleotides. Here we assume all the nascent DNA is formed from the tracer precursors supplied. Nascency is defined operationally by a fragmentation process; hence this estimate is, if anything, only the upper limit of any real unit in the cell.

The site of attachment of the "replicator" seems to be of protein nature. Treatment of the lysate before detergent treatment with pronase releases the nascent DNA which bands at a higher stratum in a sucrose gradient compared to the non-replicating DNA. The patterns are consistent with the length of exposure to H^3 thymidine. The shift in sedimentation is assumed to be due to the dissociation of the replicating point from the membrane so that the nascent DNA is of smaller molecular size compared to the old DNA. This is based on the assumption that the results obtained are not due to any configurational differences between nascent and bulk DNA to impose the observed differential mobility in a sucrose gradient. The molecular weights were calculated using T_7 DNA as standard in sucrose zone sedimentation. In E. coli the nascent DNA, enriched in the pellet, dissociates on subsequent treatment with sodium deoxycholate (Smith and Hanawalt, 1965).

In experiments of pulse labeling with H^3 uridine for 30 seconds, 25-30% of the "messenger RNA" of the cells was found in the pellet. Several authors have shown that protein synthesis occurs on the membrane of bacterial cell (see Schlessinger, 1963). In our experiments the pellet fraction was found to incorporate all 4 deoxynucleoside triphosphates using the primer already present. This is Mg^{++} dependent and requires all 4 triphosphates. The details of all these experiments mentioned above will be published elsewhere.

From the above observations, the most plausible explanation is that membrane cell wall is the site of DNA synthesis in bacteria.

The discouraging results of efforts (Richardson, et. al., 1963, etc.) to demonstrate substantial net replication of DNA with intact genetic activity through the in vitro system can probably be explained by the interruption of coherent linear replication. This results in the formation of DNA whose thermal denaturation is spontaneously reversible and which appears to have a highly involuted structure (Schildkraut, et. al., 1964). This has raised some question whether the DNA polymerase now studied in vitro is the crucial enzyme of genetic replication; however, its association with organized structures may provide the essential basis of continued orderly replication.

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